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Platelets orchestrate remote tissue damage after mesenteric ischemia-reperfusion

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Lapchak PH, Kannan L, Ioannou A, Rani P, Karian P, Lucca JJD, Tsokos GC. Platelets orchestrate remote tissue damage after mesenteric ischemia-reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G888–G897, 2012. First published February 2, 2012; doi:10.1152/ajpgi.00499.2011.—Ischemia-reperfusion (I/R) injury is a leading cause of morbidity and mortality. A functional role for platelets in tissue damage after mesenteric I/R is largely unknown. The hypothesis that mesenteric I/R local and remote injury are platelet dependent was tested. Using a murine mesenteric I/R model, we demonstrate that platelets orchestrate remote lung tissue damage that follows mesenteric I/R injury and also contribute, albeit to a lesser degree, to local villi damage. While lung damage is delayed compared with villi damage, it increased over time and was characterized by accumulation of platelets in the pulmonary vasculature early, followed by alveolar capillaries and extravasation into the pulmonary space. Both villi and lung tissues displayed complement deposition. We demonstrate that villi and lung damage are reduced in mice made platelet deficient before I/R injury and that platelet transfusion into previously platelet-depleted mice before I/R increased both villi and lung tissue damage. Increased C3 deposition accompanied platelet sequestration in the lung, which was mostly absent in platelet-depleted mice. In contrast, C3 deposition was only minimally reduced on villi of platelet-depleted mice. Our findings position platelets alongside complement as a significant early upstream component that orchestrates remote lung tissue damage after mesenteric I/R and strongly suggest that reperfusion injury mitigating modalities should consider the contribution of platelets.

ischemia-reperfusion injury; intestine; lung; complement; platelet depletion

ISCHEMIA-REPERFUSION (I/R) injury occurs as a consequence of an initial deprivation in blood flow, followed later by its return to the affected tissue. This return of circulation triggers an intense inflammatory response locally and subsequently in remote organs (20). Mesenteric I/R injury results in the loss of intestinal integrity characterized by intestinal epithelial cell damage, often accompanied by hemorrhage in the intestinal lumen (2, 36). Components from both innate and adaptive immunity, including natural immunoglobulin and complement components, as well as different leukocyte subsets, contribute to local tissue damage after I/R injury (10, 22, 28, 50, 79). A direct role for platelets in I/R injury-mediated tissue damage has not been fully investigated.

Platelets primarily function to maintain hemostasis, are key components in coagulation, and are important in host defense (37, 83). It is well recognized that platelets play a role in chronic and acute inflammatory responses due to their proinflammatory nature (6, 18, 37, 53, 66, 69). Recent reports have demonstrated that platelets activate the complement pathway, complement components can activate platelets, and platelets can become coated with C fragments (19, 34, 59–63, 66, 69). A number of clinical reports demonstrate that trauma, including burn injury and I/R injury, leads to platelet dysregulation (26, 27, 32, 42, 44, 53, 66, 69, 81). While there is ample evidence demonstrating a functional role for platelets in ischemic stroke (30, 49, 54, 64, 65), there is little published evidence supporting a pathological role for platelets in local and remote tissue damage after I/R injury.

We tested the hypothesis that platelets alone or in combination with complement mediate local and remote tissue damage after mesenteric I/R injury. In this report, we demonstrate that platelets orchestrate remote lung tissue damage after mesenteric I/R injury and also contribute, albeit to a lesser degree, to villi damage. Data presented herein position platelets alongside complement as a significant early upstream component that orchestrates remote tissue damage after mesenteric I/R.

MATERIALS AND METHODS

Mice

Adult, 8- to 12-wk-old male C57BL/6J mice were obtained (Jackson Laboratories, Bar Harbor, ME) and used for all experiments. Mice underwent at least 5 days of acclimatization before experimentation. All mice used in this study were maintained in specific pathogen-free conditions in the animal research facility at the Beth Israel Deaconess Medical Center (BIDMC).

I/R Injury Procedure

Mice were randomly assigned to sham or I/R groups ($n = 3–4$ per group). Mice were anesthetized by intraperitoneal injection of 72 mg/kg pentobarbital (Nembutal, Lundbeck, Deerfield, IL) and maintained with 36 mg/kg of pentobarbital by intraperitoneal injection. All experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the BIDMC.

A midline laparotomy was made, and mice were allowed to equilibrate for 30 min. The superior mesenteric artery was identified and isolated, and a small nontraumatic microvascular clip delivering ~85 g of pressure was applied for 30 min. The clip was removed after this ischemic phase, and the intestine was allowed to reperfuse from 1.5 to 20 h. Sham-treated mice underwent an identical surgical intervention without artery occlusion. The laparotomy incision was sutured with 4.0 SofSilk (Synture, Mansfield, MA), and the injured mice were resuscitated with 1.0 ml prewarmed sterile PBS subcutaneously and monitored during the reperfusion period. Body temperature was maintained at 37°C throughout the preparatory and experimental procedure on a temperature-controlled heating pad.

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At the conclusion of the reperfusion period, mice were euthanized by carbon dioxide asphyxiation, following the IACUC Guidelines of the BIDMC. The small intestine was isolated, and a 20-cm section distal to the gastroduodenal junction was removed and flushed with ice-cold PBS, followed by ice-cold 10% phosphate-buffered formalin before overnight fixation in 10% phosphate-buffered formalin at 4°C. Lung removal consisted of intact extraction of the bronchial tree after expansion with tracheal administration of 200–300 μl of ice-cold 10% phosphate-buffered formalin and fixed overnight in 10% phosphate-buffered formalin at 4°C. Formalin-fixed intestine and lung tissues were washed extensively in PBS, processed, and embedded in paraffin for histological and immunohistochemical analysis.

Histology

Histopathology was determined on formalin-fixed paraffin embedded 5- to 6-μm sections stained with hematoxylin and eosin using standard methods and examined under light microscope.

Intestinal villi injury scoring. Fifty to one hundred villi per tissue section were graded using a 6-tiered scale, as previously described by Chui et al. (11), and the mean score was recorded in a third-party blinded fashion. Briefly, normal appearing villus was assigned a score of 0, while villi demonstrating tip distortion were scored as 1. Villi without goblet cells and with Guggenheims' spaces were scored as 2, and villi containing patchy disruption of the epithelial cells were scored as 3. Villi demonstrating exposed, intact lamina propria and sloughing of epithelial cell were scored as 4. Villi demonstrating exuding lamina propria were assigned a score of 5, and, last, villi with hemorrhage or denudation were scored as 6.

Lung injury scoring. Alveolar and periluminal injury scores for each lung section were calculated based on Cooke's method (17), and the mean score was recorded in a third-party blinded fashion. Ten to twenty fields at high-power field magnification (×400) were viewed for each lung section and scored for alveolar infiltration on a 3-tiered scale. The following calculation for alveolar scores was performed as follows: a score of 0 was given when no infiltrate was present; a score of 1 was given when the infiltrate could be visualized easily only at ×400; when infiltrates were readily visible, a score of 2 was assigned; and the score for consolidation was 3. Similarly, each section was scored for periluminal damage (airway or blood vessel) at ×100. The calculation for periluminal scores was as follows: when there was no infiltrate, a score of 0 was assigned; when the infiltrate was between 1 and 3 cell layers thick, the score was 1; for infiltrates ranging from 4 to 10 cells layers thick, a score of 2 was assigned; and infiltrates >10 cell layers thick were scored as 3. Based on the overall involvement of the section, a severity score was calculated: the severity score for 0–25% involvement was 1; a severity score of 2 was assigned for 25–50% involvement; and the severity score for >50% involvement was 3. For calculation of the total lung injury score, the means of alveolar and periluminal scores for each section were summed up and multiplied by the severity score, which gave a final score ranging from 0 to 18.

Platelet Depletion

Two days before I/R injury, mice received a single intraperitoneal injection of a titred affinity purified endotoxin-free rabbit anti-mouse polyclonal antibody (α-PLT-Ig) at a final concentration of 2.5 mg/kg body wt. It was prepared in house with commercially available rabbit anti-mouse platelet antiserum (Inter-Cell Technologies, Jupiter, FL) and was affinity purified on a protein G column (Pharmacia, Uppsala, Sweden) (31).

Platelet Preparation and Transfusion Protocol

For platelets, mice were bled via cardiac puncture (~500–800 μl per bleed) into 1 ml syringe containing 500 μl of citrate-anticoagulant, and then the circulated blood was transferred to a tube containing 5 ml PIPES buffer. The blood was pooled and centrifuged at 120 *g*, and the platelet-rich plasma was aspirated off; care was taken not to disturb or aspirate the buffy coat. To the platelet-rich supernatant 1 μM of PGE_1 and 1 U/ml of apyrase (Sigma, St. Louis, MO) were added and centrifuged again at 1,000 *g* for 10 min. The platelet pellet was resuspended in HEPES-modified Tyrode's buffer. Platelet numbers were determined using Hemavet 850 (Drew Scientific, Farmington, CT) and were adjusted to 2×10^9/ml.

Each transfusion protocol began with groups of mice pretreated with control-Ig or α-PLT-Ig 48 h before sham or I/R injury. Two mouse equivalents of platelets were transfused fresh via cardiac puncture within 1 h of collection. At the conclusion of the reperfusion period, organs were collected by the procedure described above.

Immunohistochemistry

To perform immunohistochemical staining, formalin-fixed paraffin sections of intestine and lung were subjected to rehydration and antigen retrieval using a standard protocol. For immunohistochemical studies, the following reagents were used: platelet (affinity-purified rabbit polyclonal antibody) and rabbit anti-mouse C3 (B-9) (Santa Cruz, CA). Peroxidase-conjugated affinity-purified secondary antibodies to rabbit immunoglobulin were purchased from Jackson ImmunoResearch. Stained sections were developed with NovaRed (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA). Appropriate isotype controls were used.

Flow Cytometry

Whole blood was obtained from experimental mice that either underwent 30 min of ischemia and 3 h of reperfusion, or mock injury (sham controls) ($n = 4$ per group). The blood was incubated with antibodies to C3 and the platelet marker CD61 (Millipore, Billerica, MA), and then samples were evaluated by flow cytometry. The analysis was performed on the LSRII flow cytometer (BD Biosciences, San Jose, CA), and the events were analyzed using FlowJo software version 7.6.1 (TreeStar, Ashland, OR).

Image Development and Handling

Histological and immunocytochemistry slides were viewed on a Nikon Eclipse 80i microscope, and images were taken using the Nikon DS-FiI digital camera and saved as tiff files. Images were adjusted globally using the adjustment feature in the RGB channel using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

Statistical Analysis

GraphPad Prism 4.0 for Windows software program (GraphPad Software, San Diego, CA) was used for all statistical calculations. All data are presented as mean ± SD. The comparison of sham controls and experimental injury data was analyzed using the nonparametric Mann-Whitney *t*-test for unpaired samples. Differences were considered significant when the P value was ≤0.05.

RESULTS

Role of Platelets and Complement in Time-dependent Local and Remote Tissue Damage After Mesenteric I/R

First we determined whether platelets contribute to villi and lung damage, in a reperfusion time-dependent experiment by immunohistochemistry. Moderate staining for platelets was found at the tips of damaged villi early at 1.5 h and up to 6 h reperfusion, suggesting that platelets may not be the primary contributor to villi damage in mesenteric I/R injury (Fig. 1, *E–G*). Platelet staining steadily increased in lungs with reperfusion time, as shown in Fig. 1, *M–P*. Platelets were observed

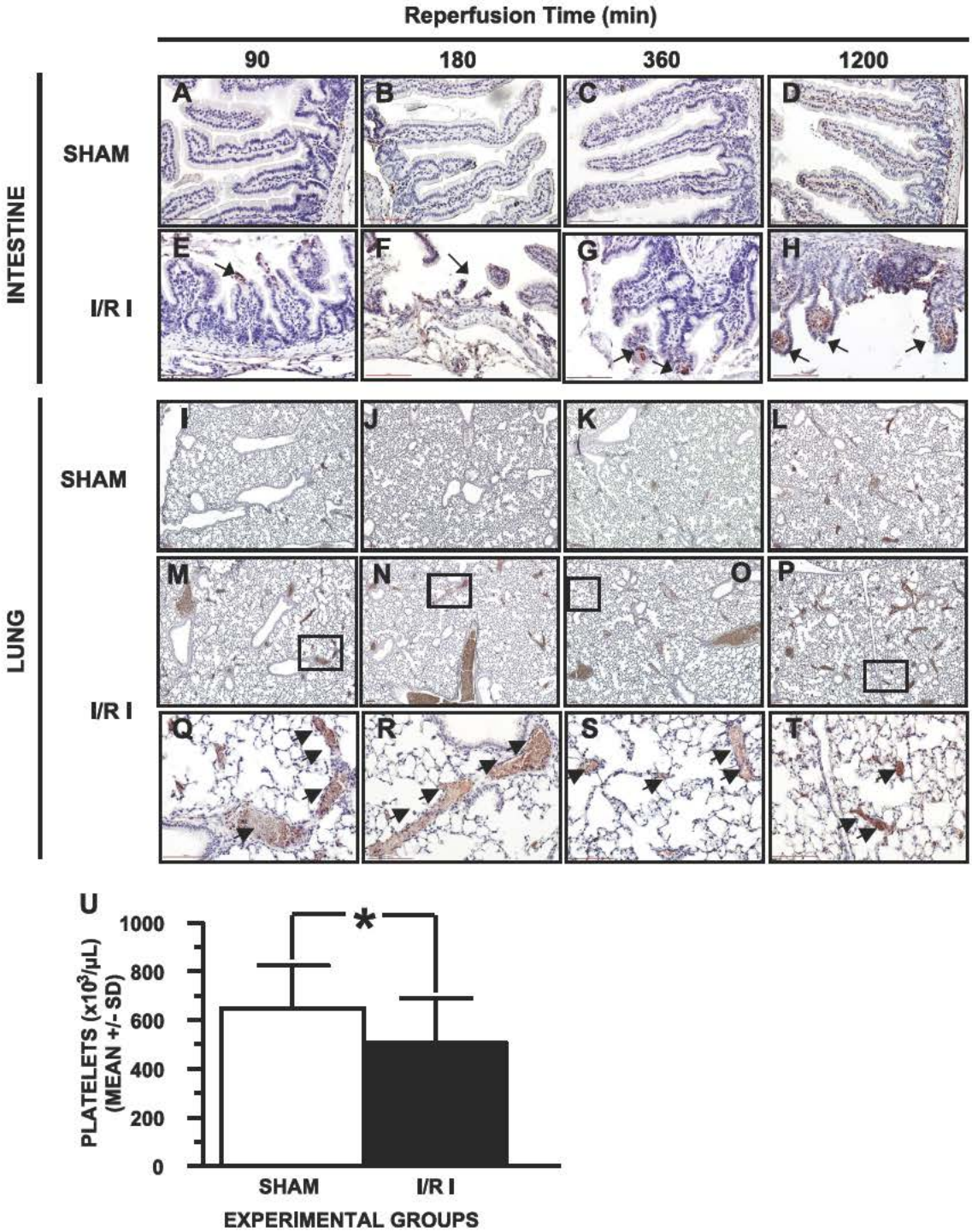

Fig. 1. Immunohistochemical identification of platelets in villi and lung tissue after mesenteric ischemia and reperfusion. Tissue sections of intestine after 30 min of mesenteric ischemia, and 1.5 h (*E*), 3.0 h (*F*), 6.0 h (*G*), and 20 h (*H*) of reperfusion, and time-matched sham controls (*A–D*) were stained for platelets (red) and counterstained with hematoxylin (blue). Images are representative of 3–4 mice per group. Images are ×200 magnification for experimental and time-matched sham controls. Tissue sections of lung after 30 min of mesenteric ischemia, and 1.5 h (*M* and *Q*), 3.0 h (*N* and *R*), 6.0 h (*O* and *S*), and 20 h (*P* and *T*) of reperfusion, and time-matched sham controls (*I–L*) were stained for platelets (red) and counterstained with hematoxylin (blue). Images are representative of 3–4 mice per group. Images are ×200 magnification for experimental and time-matched sham controls. Arrows indicate areas of positive staining. *U*: circulating platelet numbers were determined after 3-h reperfusion and compared with time-matched sham controls. I/R I, ischemia-reperfusion injury. Boxes in *M–P* indicate lung sections expanded as shown in *Q–T*. *$P \leq 0.05$ for I/R I compared with sham controls.

at 1.5-h reperfusion in the pulmonary venules (Fig. 1*M*), followed by additional venules, arterioles, as well as alveolar capillaries at later reperfusion times (Fig. 1, *N–P*) compared with sham-operated animals (Fig. 1, *I–L*). We also determined circulating platelet numbers at 3-h reperfusion and found a modest thrombocytopenia (Fig. 1*U*).

Next we evaluated complement levels by immunohistochemistry as it relates to villi and lung damage. Intestine and

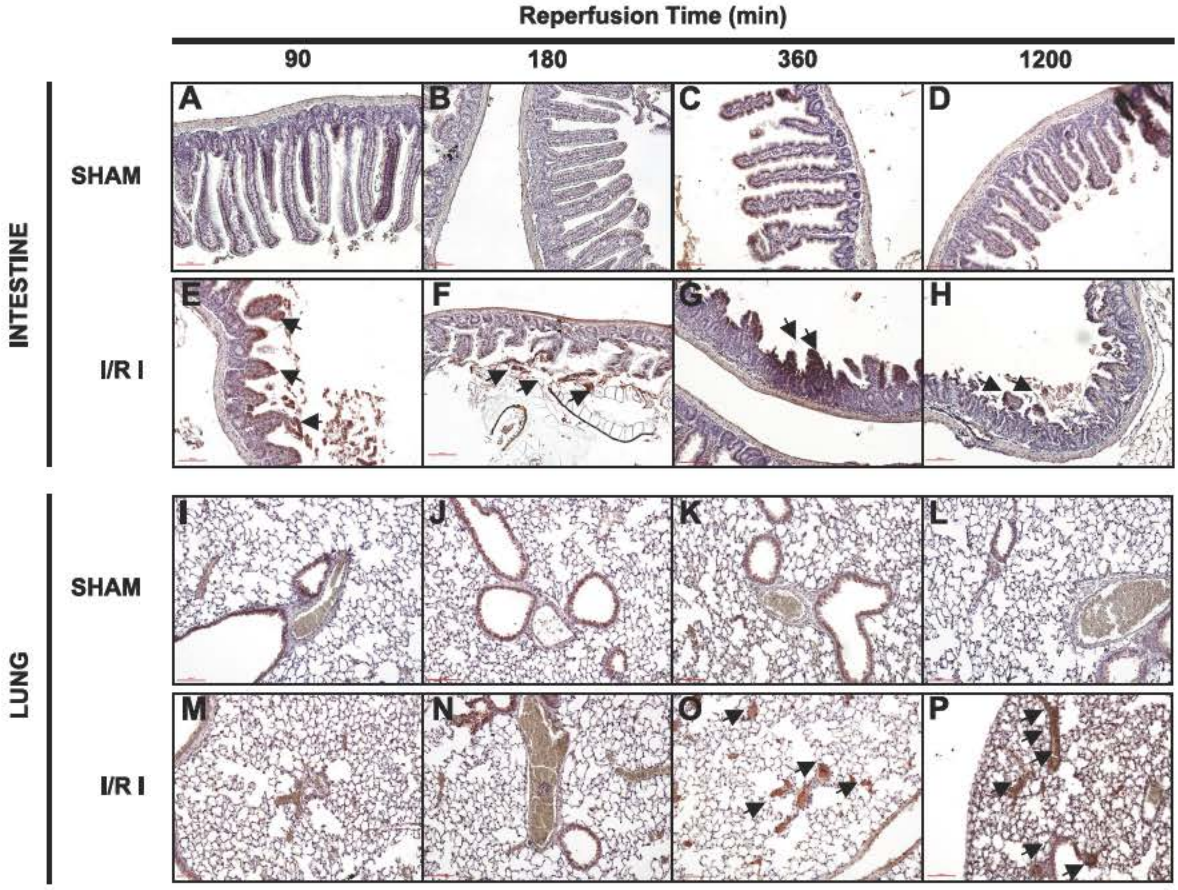

Fig. 2. Immunohistochemical identification of C3 in villi and lung tissue after mesenteric ischemia and reperfusion. Tissue sections of intestine after 30 min of mesenteric ischemia, and 1.5 h (*E*), 3.0 h (*F*), 6.0 h (*G*), and 20 h (*H*) of reperfusion, and time-matched sham controls (*A–D*) were stained for C3 (red) and counterstained with hematoxylin (blue). Images are representative of 3–4 mice per group. Images are ×100 magnification for experimental and time-matched sham controls. Tissue sections of lung after 30 min of mesenteric ischemia, and 1.5 h (*M*), 3.0 h (*N*), 6.0 h (*O*), and 20 h (*P*) of reperfusion, and time-matched sham controls (*I–L*) were stained for C3 (red) and counterstained with hematoxylin (blue). Images are representative of 3–4 mice per group. Images are ×100 for experimental and time-matched sham controls. Arrows indicate areas of positive staining.

lung tissues were stained for complement C3, which recognizes C3 precursor, C3a anaphylatoxin, C3 α-chain, C3 β-chain, and C3b α-chain. In contrast to platelets, C3 deposition was extensive on villi at all reperfusion time points and was found on all damaged villi observed (Fig. 2, *E–H*). Lung tissue displayed C3 deposition in the vasculature, which occurred prominently only after 6-h reperfusion (Fig. 2, *O* and *P*).

After mesenteric I/R, we observed a steady increase in platelet staining as reperfusion time increases, which preceded the appearance of C3 deposition in the vasculature, and lung tissue that increased starting at 6-h reperfusion. Platelet sequestration in the pulmonary vasculature supports our finding of reduced circulating platelets after mesenteric I/R (Fig. 1*U*) and suggests that activated platelets may deposit complement components from the primary site of injury to remote tissues. Platelets may also activate the complement pathway at remote sites to initiate de novo tissue damage.

Isolated Platelets Are Decorated With Complement

Next we determined whether circulating platelets may become decorated with complement components after localized trauma (Fig. 3). As shown in a representative sample, >80% of platelets ($CD61^+$) were $C3^+$ (Fig. 3, *right*), contrasting the <10% of platelets that are $C3^+$ in the sham controls (Fig. 3, *left*).

Platelet Deficiency Reduces Local and Remote Tissue Damage: Changes in Complement Deposition in Remote Tissue

To further establish a role for platelets in tissue damage after mesenteric I/R, the level of villi and lung tissue damage after

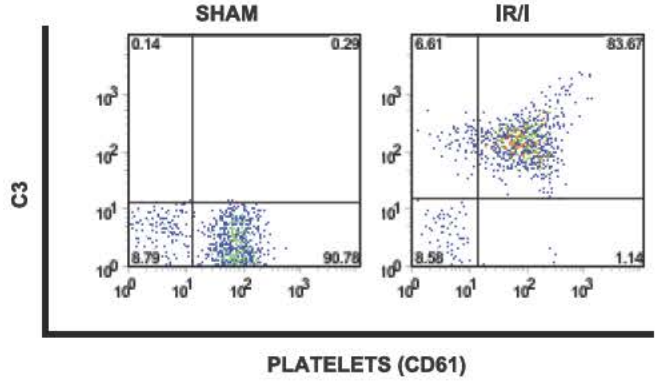

Fig. 3. Flow cytometry of platelets after mesenteric ischemia and reperfusion. Whole blood was isolated by cardiac puncture after 30 min of ischemia and 3 h of reperfusion and stained for complement component 3 (C3) and for the platelet marker CD61. Plots are representative of 4 mice per sham and I/R I group.

30 min of mesenteric ischemia and 3 h of reperfusion (Fig. 4) from platelet-intact, from platelet-deficient, and from platelet-transfused mice that were depleted of platelets were compared using histological analysis. Mice made platelet deficient about 2 days before mesenteric I/R displayed a modest but significant reduction in villi damage compared with platelet-intact mice (Fig. 4, *B* and *F*) and was confirmed by histological scoring (Fig. 4*M*). Platelet numbers were determined in platelet-deficient mice and were in the range of 20–25% of normal platelet numbers (Fig. 4*O*), sufficient to prevent experimental stressed-induced hemorrhage. Next platelet-deficient mice were transfused with platelets, the numbers of which are sufficient to return those mice to ≥90% of normal platelet levels; villi damage was again increased to levels before depletion (Fig. 4*J*) and was confirmed by histological scoring (Fig. 4*M*). This further supports our findings that platelets may contribute to, but perhaps not be central to, villi damage.

We wanted to determine whether platelets were central to remote organ (lung) tissue damage after mesenteric I/R. Lung damage from platelet-intact, platelet-depleted, and from platelet-transfused mice after 30 min of mesenteric ischemia and 3 h of reperfusion were compared (Fig. 4, *C*, *D*, *G*, *H*, *K*, *L*, and *N*). Lung damage after mesenteric I/R in platelet-deficient mice was significantly reduced compared with that in platelet-intact mice (Fig. 4, *D* and *H*). Lung damage in platelet-transfused mice was significantly increased and resembled that observed in platelet-intact mice (Fig. 4, *D* and *L*). These findings establish for the first time firmly that platelets directly mediate remote lung damage following mesenteric ischemia and reperfusion.

To further establish this novel role for platelets as a direct (lung) or an indirect (intestine) mediator of tissue damage, lung and intestine from platelet-intact, platelet-deficient, and platelet-transfused mice were stained for platelets (Fig. 5). Platelets were present in the lungs of platelet-intact mice and in platelet-

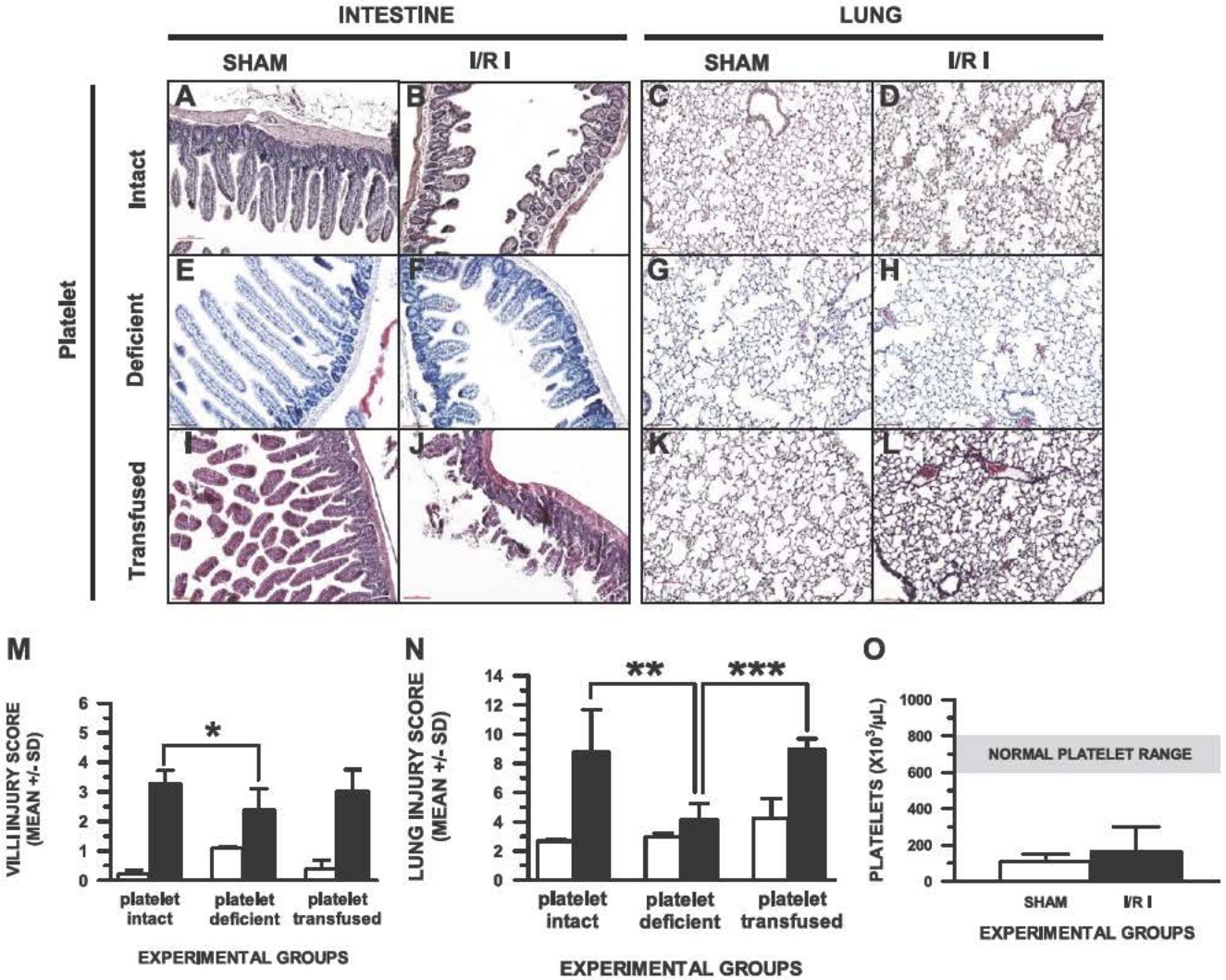

Fig. 4. Comparative differences in the small intestine and lung from platelet-intact, platelet-deficient, and platelet-reconstituted mice after I/R I. Hematoxylin- and eosin-stained sections of mouse small intestine after 30 min of ischemia and 3.0 h reperfusion are shown. Images are representative of 3–4 mice per group in 2 experiments. Images of intestinal villi from platelet-intact sham (*A*) and I/R I (*B*), platelet-deficient sham (*E*) and I/R I (*F*), and platelet-transfused sham (*I*) and I/R I (*J*) mice are shown. Images of lung from platelet-replete sham (*C*) and I/R I (*D*), platelet-deficient sham (*G*) and I/R I (*H*), and platelet-transfused sham (*K*) and I/R I (*L*) mice are shown. All images shown are ×100 magnification. Injury score (mean ± SD) in intestine (*M*) and lung (*N*) comparing sham (open bars) vs. I/R I (solid bars) is shown. *O*: circulating platelet numbers in platelet-depleted mice after 3-h reperfusion compared with time-matched sham controls. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ for I/R I compared with sham controls.

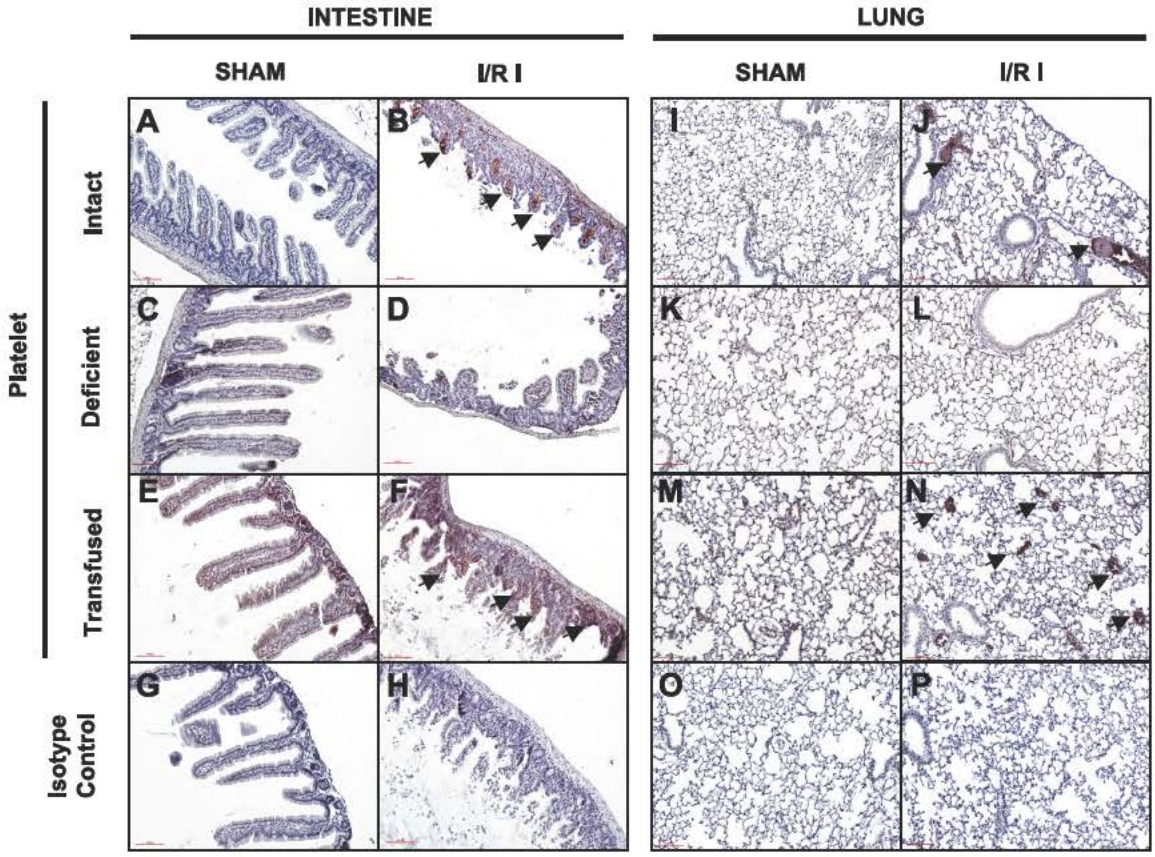

Fig. 5. Immunohistochemical identification of platelets in intestine and lung after mesenteric ischemia and reperfusion in platelet-intact, platelet-deficient, and platelet-reconstituted mice. Tissue sections of mouse small intestine and lung after 30 min of ischemia and 3.0 h of reperfusion are shown. Images are representative of 3–4 mice per group in 2 experiments. Sections were stained for platelet (red) and counterstained with hematoxylin (blue) of intestinal villi from platelet-intact sham (*A*) and I/R I (*B*), platelet-deficient sham (*C*) and I/R I (*D*), and platelet-transfused sham (*E*) and I/R I (*F*) mice. Images of isotype controls from sham (*G*) and I/R I intestine (*H*) are shown. Images of lung from platelet-replete sham (*I*) and I/R I (*J*), platelet-deficient sham (*K*) and I/R I (*L*), and platelet-transfused sham (*M*) and I/R I (*N*) mice are shown. Images of isotype controls from sham (*O*) and I/R I lung (*P*) are shown. All images shown are ×100 magnification. Arrows indicate areas of positive staining.

transfused mice after mesenteric I/R (Fig. 5, *J* and *N*), whereas they were virtually absent in platelet-deficient mice (Fig. 5*L*). These findings further establish that platelets are central for the induction of remote (lung) tissue damage after mesenteric I/R. Platelets were identified in the villi of platelet-intact mice and from platelet-transfused mice after mesenteric I/R (Fig. 5, *B* and *F*) and contrasts the levels observed in platelet-deficient mice (Fig. 5*D*), suggesting that platelets may play a role in villi damage after mesenteric I/R.

We next determined whether C3 deposition followed a pattern similar to that observed for platelets to establish, at least in the lung, that platelets may drive complement pathway activation, leading to remote tissue damage after mesenteric I/R (Fig. 6). Immunohistochemical identification of C3 was performed in lung and intestine from platelet-intact, platelet-deficient, and platelet-transfused mice. C3 deposition was dramatically reduced in the lungs from mice made platelet deficient before mesenteric I/R compared with lungs of platelet-intact mice (Fig. 6, *J* and *L*), and was reestablished in platelet-depleted mice transfused with syngeneic platelets (Fig. 6*N*). C3 staining was similar in platelet-transfused and platelet-intact mice (Fig. 6, *J* and *N*). C3 deposition was reduced but not absent on villi from mice made platelet deficient before I/R (Fig. 6*D*), was increased in platelet-depleted mice transfused with syngeneic platelets before I/R (Fig. 6*F*), and was similar to platelet-intact mice (Fig. 6*B*). Together, these findings suggest that platelets orchestrate lung damage directly or by driving complement pathway activation and generation of cytolytic complement fragments de novo. In contrast, platelets contribute, albeit indirectly, to the development of intestinal tissue damage following mesenteric I/R.

DISCUSSION

Although the primary function of platelets is to maintain hemostasis, platelets also contribute to pathological and immunopathological conditions due to their proinflammatory nature (6, 18, 37, 52, 53, 66, 69, 73). Platelets have been shown to play an inflammatory role in autoimmune diseases, such in rheumatoid arthritis, inflammatory bowel disease (1, 13–15, 40, 41, 52, 71–73, 76, 82), and in ischemic stroke (30, 49, 54, 64, 65), either directly or through release of their granule contents. In addition, platelets have been shown to modulate both innate and adaptive immune responses by their ability to interact with neutrophils, monocytes, and T and B lymphocytes (3, 16, 24, 25, 39, 45, 55).

Innate and adaptive immune factors, such as natural IgM, complement, T and B cells, and neutrophils have been shown

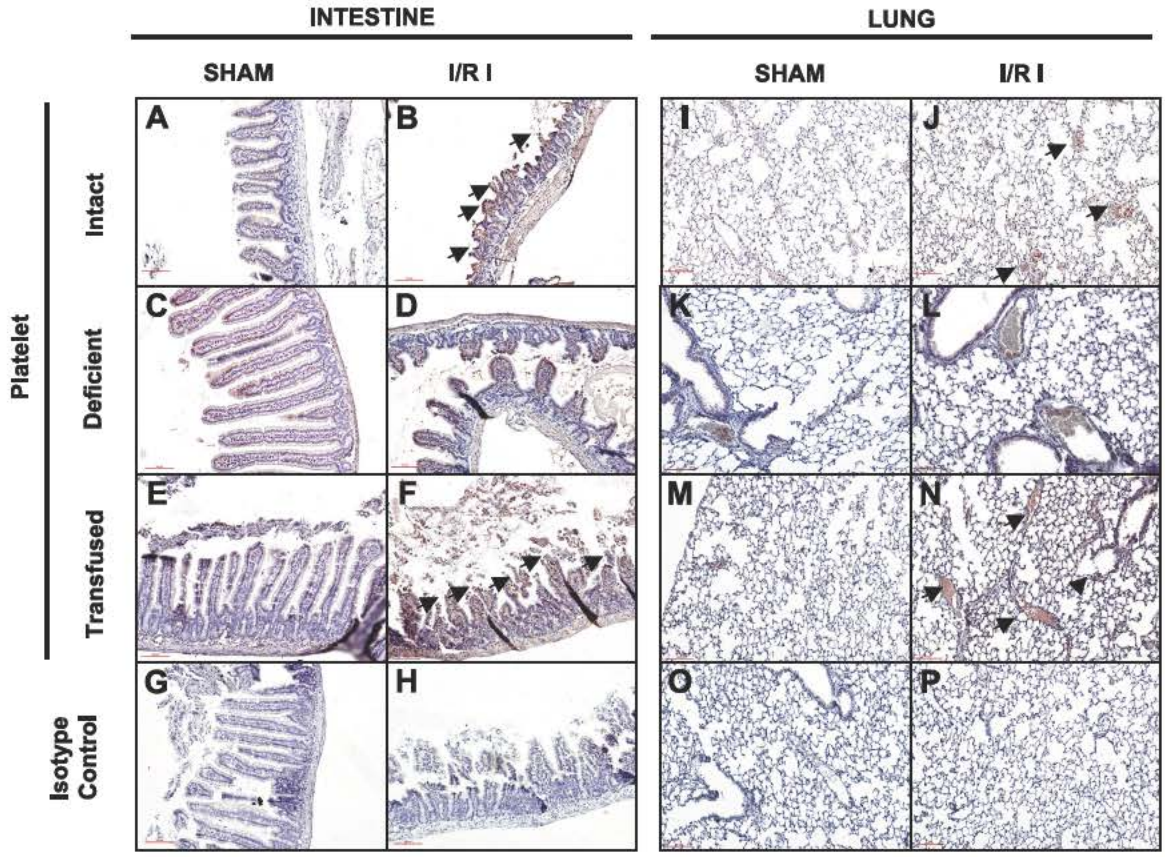

Fig. 6. Immunohistochemical identification of C3 in intestine and lung after mesenteric ischemia and reperfusion in platelet-intact, platelet-deficient, and platelet-reconstituted mice. Tissue sections of mouse small intestine and lung after 30 min of ischemia and 3.0 h of reperfusion are shown. Images are representative of 3–4 mice per group in 2 experiments. Tissues were stained for C3 (red) and counterstained with hematoxylin (blue) of intestinal villi from platelet-replete sham (*A*) and I/R I (*B*), platelet-deficient sham (*C*) and I/R I (*D*), and platelet-transfused sham (*E*) and I/R I (*F*) mice. Images of isotype controls from sham (*G*) and I/R I intestine (*H*) are shown. Images of lung from platelet-replete sham (*I*) and I/R I (*J*), platelet-deficient sham (*K*) and I/R I (*L*), and platelet-transfused sham (*M*) and I/R I (*N*) mice are shown. Images of isotype controls from sham (*O*) and I/R I lung (*P*) are shown. All images shown are ×100 magnification. Arrows indicate areas of positive staining.

to contribute to I/R injury (10, 16, 22, 29, 79, 84–86). A deficiency in T or B cells, depletion of complement with cobra venom factor, or inhibiting complement activity with sCD55 or CrIg significantly reduces, but does not completely ameliorate, intestinal villi damage after mesenteric I/R (4, 8, 9, 33, 77, 78, 80). A role for platelets in I/R-mediated tissue damage has not been fully investigated, although a recent report demonstrates that I/R injury led to dysfunctional activity of platelets (81).

The present study determined whether platelets either directly or indirectly play a role in tissue damage locally in the intestine or in remote organs after mesenteric I/R. This was accomplished by performing a time course study in which mice underwent 30 min of ischemia and 1.5–20 h of reperfusion. We evaluated tissue damage over time in both intestine and lung by standard histology (data not shown) and extended these findings by identifying platelets and C3 by immunohistochemistry. Platelets increased in the villi with the duration of reperfusion time and were most prominent between 6 and 20 h of reperfusion. This increase in tissue damage was also associated with increased C3 deposition in the villi, which was observed at the earliest time point of 1.5-h reperfusion. These findings confirm previous reports supporting a role for complement, but also newly establishes a role for platelets, albeit to a lesser degree, in villi damage after I/R (20, 29, 38).

Next we established the role of platelets in remote tissue damage after mesenteric I/R. Significant platelet sequestration in the vasculature was observed in the lung tissue in the same time course experiment. It is not clear why platelets would preferentially traffic to the pulmonary environment. While the pulmonary vasculature is heterogeneous in nature, we could speculate that the pulmonary endothelium may preferentially express chemokine receptors at higher levels than the vasculature in other organs. This higher expression may be sufficient to trap activated platelets. Alternatively, local ischemia may induce the release of products from tissues that preferentially upregulate cell surface molecules on pulmonary endothelium, leading to preferential binding of platelets (23, 56, 58).

Within the lung, predominantly monocytes and neutrophils were found within the areas of platelet accumulation (data not shown). These findings suggest that platelets may be central to remote tissue damage where they are responsible for recruiting monocytes and neutrophils to new sites of inflammation, and for potentially inducing vascular leakage by their interaction with endothelial cells lining blood vessels. This is supported by the ability of activated platelets to express CD40, CD154, CD62P, and CD162 (P-selectin glycoprotein ligand-1): these molecules can bind their respective ligands on not only neutrophils and monocytes, but also vascular endothelial cells (7,

21, 46, 48, 51, 57, 67, 74). Blocking of integrins and ligands with antibodies reduced I/R damage (70). Activated platelets can further activate trafficking platelets through similar mechanisms (21, 48, 53). Once activated, platelets release the contents of their α-granules, which are rich in proinflammatory, chemotactic, and adhesion molecules, of their dense bodies, which are a rich source of ATP and ADP, serotonin, and polyphosphates, and of their lysozomes, which are metalloproteinase rich. α-Granule products from activated platelets lead to initial expression of CD154, CD62Pm and CD162 on their surfacesm which are later cleaved by the metalloproteinases that are released from platelet lysozomes (5, 68, 75). Release of platelet lysozome contents into areas of newly established inflammation may also be important in the development of vascular leakage through the breakdown of tight junctions and basement membranes. Hence, these soluble platelet products may be sufficient to initiate inflammation (43). These wide-ranging activities of platelets are in line with our observed findings of lung damage. Furthermore, our immunohistochemical findings support our observation that circulating platelet numbers are decreased at 3-h reperfusion. Circulating platelets likely increase in response to a positive feedback mechanism and are released from the spleen, where 30% of the total circulating platelets reside (31); this would explain why the reduction in circulating platelet numbers is modest, yet significant, compared with sham controls at this time point.

Intense positive staining for C3 in the lung, unlike in the intestine, which was already apparent at 1.5 h of reperfusion, was detected later, from 6 to 20 h after reperfusion. C3 may bind to activated platelets, which, in turn, may facilitate binding of activated C3-coated platelets to complement receptor 1 on pulmonary vascular endothelium (12, 35, 47). However, flow cytometry demonstrated C3 deposition on platelets obtained from mice after 3 h of reperfusion. While it is possible that platelets transport complement to the lung, our immunohistochemical findings do not support this. However, our findings do support the notion that activated platelets can initiate the complement pathway, which is now well documented (59–62). Furthermore, local platelet activation in the intestine and within the villi can occur via complement fragments, which is also now well documented (63, 66, 69). This is in agreement with our immunocytochemical findings.

C57BL/6J mice were made deficient in platelets before mesenteric I/R to further address the intricate role that platelets play in tissue damage after mesenteric I/R. Platelet values in these mice were maintained to ~15–20% of normal platelet values, so as not to introduce hemorrhage as a confounding factor. Platelet-deficient mice that underwent mesenteric I/R had both a significant decrease in villi and lung damage. Although villi damage was reduced, it was not completely abolished. Staining for C3 in intestine from platelet-deficient mice that underwent mesenteric I/R revealed C3 levels similar to that observed with platelet-intact mice, suggesting that complement is a dominant contributor to villi damage and acts upstream of platelets. In contrast, lung damage in platelet-deficient mice was virtually absent and was associated with minimal C3 deposition. These findings support our hypothesis that lung tissue damage was mediated by platelets that acted upstream of C3 and suggests that platelets may initiate complement activation locally at sites of inflammation.

The central role of platelets in the induction of remote tissue damage after mesenteric I/R was further confirmed by using mice depleted of platelets that were transfused with syngeneic platelets on the day of I/R procedure. Mesenteric I/R in mice transfused with platelets to normal values led in increased villi damage. Villi damage increase was not statistically significant, although it did trend to levels (by injury score) of platelet-intact mice. Complement staining was similar in both platelet-intact and platelet-deficient mice. These findings further support a secondary role for platelets in villi damage after I/R. In contrast, lung tissue damage in platelet-transfused mice was significantly increased compared with that in platelet-deficient mice and similar to levels observed in platelet-intact mice where lung damage scores were comparable. C3 staining was again apparent and increased in lungs from platelet-transfused mice after mesenteric I/R.

Previous studies have focused predominantly on innate and adaptive mechanisms of tissue damage after I/R that included complement, natural Ig, lymphocyte subsets, and neutrophils, with little attention to platelets. Local tissue damage and initiation of remote tissue damage after mesenteric I/R are complex in nature and involve many contributing factors. However, it was not clearly understood by which mechanisms remote tissue damage was initiated following local trauma. Here, platelets were identified as being a critical and central mediator of remote tissue damage and as a secondary component in local damage. In local trauma, platelets play a less significant role, although they contribute to local villi damage. Thus, preventing or inhibiting remote tissue damage may be possible by targeting platelets with currently available compounds that specifically inhibit proinflammatory functions of platelets.

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DISCLAIMER

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting the views of the United States Department of the Army or the Department of Defense.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.H.L., J.J.D.L., and G.C.T. conception and design of research; P.H.L., P.R., and P.K. performed experiments; P.H.L., L.K., A.I., and G.C.T. analyzed data; P.H.L., L.K., A.I., and G.C.T. interpreted results of experiments; P.H.L. and L.K. prepared figures; P.H.L. and G.C.T. drafted manuscript; P.H.L., L.K., A.I., P.R., P.K., J.J.D.L., and G.C.T. edited and revised manuscript; P.H.L., L.K., A.I., P.R., P.K., J.J.D.L., and G.C.T. approved final version of manuscript.

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